Cardiac remodeling and genotype-specific pathogenic effects in dilated cardiomyopathy

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LMNA-related cardiac disease: Late onset with a variable and mild phenotype in a large cohort of patients with the *LMNA p.* (Arg331Gln) founder mutation

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Abstract

Background

Interpretation of missense variants can be especially difficult when the variant is also found in control populations. This is what we encountered for the *LMNA* c.992G>A (p.(Arg331Gln)) variant. Therefore, to evaluate the effect of this variant, we combined an evaluation of clinical data with functional experiments and morphological studies.

Methods and Results

Clinical data of 23 probands and 35 family members carrying this variant were retrospectively collected. A time-to-event analysis was performed to compare the course of the disease with carriers of other *LMNA* mutations. Myocardial biopsies were studied with electron microscopy (EM) and by measuring force development of the sarcomeres. Morphology of the nuclear envelope was assessed with immunofluorescence on cultured fibroblasts.

The phenotype in probands and family members was characterized by atrioventricular conduction disturbances (61% and 44%, respectively), supraventricular arrhythmias (69% and 52%, respectively) and dilated cardiomyopathy (74% and 14%, respectively). *LMNA* p.(Arg331Gln) carriers had a significantly better outcome regarding the composite endpoint (malignant ventricular arrhythmias, end stage heart failure or death) compared to carriers of other pathogenic *LMNA* mutations. A shared haplotype of 1 Mb around *LMNA* suggested a common founder. The combined LOD score was 3.46. Force development in membrane-permeabilized cardiomyocytes was reduced due to decreased myofibril density. Structural nuclear *LMNA*-associated envelope abnormalities, i.e. blebs, were confirmed by EM and immunofluorescence microscopy.

Conclusion

Clinical, morphological, functional, haplotype and segregation data all indicate that *LMNA* p.(Arg331Gln) is a pathogenic founder mutation with a phenotype reminiscent of other *LMNA* mutations, but with a more benign course.

Key words:

dilated cardiomyopathy; atrioventricular block; atrial fibrillation; lamin A/C; survival

Introduction

The *LMNA* gene encodes for the intermediate filament proteins lamin A and C. *LMNA* mutations are associated with a wide spectrum of phenotypes ranging from progeroid syndromes, muscular disease and lipodystrophy to isolated cardiac disease (dilated cardiomyopathy (DCM), conduction disorders) and phenotypes consisting of combinations of these different features[1]. Although their precise role is unknown, *LMNA* proteins are believed to play an important role in the structural integrity of the cell nucleus and in gene regulation[2].

LMNA is one of the genes most frequently involved in genotyped DCM[3]. Sinus node dysfunction, atrioventricular conduction disorders, supraventricular and ventricular arrhythmias often precede or accompany DCM[4]. *LMNA*-related cardiac disease is associated with a high incidence of major cardiac events like sudden cardiac death (SCD), appropriate implantable cardioverter-defibrillator (ICD) therapy or end stage heart failure. DCM patients with an *LMNA* mutation are, in general, believed to have a poor prognosis compared with non-*LMNA*-mutation DCM patients[56].

Currently, with all the new DNA sequencing technologies implemented in routine patient care, increasing numbers of DNA variants are being identified[7]. Classifying a variant as "pathogenic" has important implications for genetic counselling, the identification of family members at risk, clinical management and sometimes even clinical risk-stratification[8]. However, assigning "pathogenicity" to a variant is often challenging, particularly when the variant is found in ostensibly healthy controls.

In the GoNL database, which contains genome sequencing data of approximately 500 unrelated Dutch subjects, *LMNA* c.992G>A (p.(Arg331Gln)) was found four times, but in low quality calls. We therefore had difficulty assigning the correct label to this variant. Although in silico prediction models predicted that this substitution is deleterious or probably damaging, we sought to find additional evidence for the potential pathogenicity of this mutation by evaluating clinical data, studying morphology of the nuclear envelope and analyzing functional effects on the myocytes and fibroblasts of mutation carriers.

Methods

Mutation analysis

Probands with the *LMNA* p.(Arg331Gln) variant were identified by next generation sequencing (NGS) using targeted panels (the list of genes screened is available on request) or by Sanger sequencing[9,10]. Variants found with NGS were confirmed by Sanger sequencing. Variants in family members were analyzed by Sanger sequencing. Written

informed consent was obtained from all participants according to the local medical ethics committees of our hospitals.
The online Genome of the Netherlands (GoNL) database and the Exome Aggregation Consortium dataset were searched for the *LMNA* p.(Arg331Gln) variant to check for its presence in the general population[11,12].

Clinical evaluation

We collected retrospective clinical data on 23 probands (A to W), who carried the *LMNA* p.(Arg331Gln) variant and on 35 family members carrying this variant. When available, data on medical history, physical examination, 12 lead electrocardiogram (ECG), 24-hour ambulatory ECG (Holter) and/or exercise-ECG (X-ECG), transthoracal echocardiography, magnetic resonance imaging (MRI) of the heart, myocardial perfusion scintigraphy and coronary angiography were collected. In cases of atrial fibrillation (AF) or pacemaker (PM) rhythm, earlier ECGs were analyzed for conduction disorders.
Pedigrees of the families were constructed to study segregation of the variant with the phenotype. Family members were considered to have cardiac involvement if there was evidence of sinus node dysfunction, supraventricular and ventricular arrhythmias, atrioventricular and ventricular conduction delay, PM and/or ICD implantation, structural cardiac abnormalities (determined by echocardiography or MRI) or symptomatic heart failure in the absence of other known causes. For a more detailed description of the phenotypes and definitions used see Supplemental Materials.

Time-to-event analysis

Data of 56 carriers of a pathogenic *LMNA* mutation – not p.(Arg331Gln) – associated with DCM (31 probands and 25 family members) were used to compare outcome. Pathogenicities of these mutations were previously assessed using a clinical classification scheme described earlier[13]. Data of these *LMNA* mutation carriers have been analyzed and used in previous studies[6,13,14]. An event was defined as a composite of the following endpoints: appropriate ICD therapy, out of hospital cardiac arrest (OHCA), heart transplant/left ventricular assist device implantation (HTx/LVAD) or death. Appropriate ICD therapy was defined as antitachycardia pacing (ATP) or an ICD discharge for termination of ventricular tachycardia or fibrillation. A log-rank test was performed to evaluate the potential difference in outcome between the *LMNA* p.(Arg331Gln) mutation carriers and other *LMNA* mutation carriers, labeled "*LMNA* group". We also compared the outcome of p.(Arg331Gln) carriers to those of individuals with only a missense *LMNA* mutation (16 probands and 11 family members) from the "*LMNA* group", labeled "*LMNA* missense only".

Haplotype analysis and genealogy

To evaluate whether the mutation originated from a common founder, 12 microsatellite markers around *LMNA* were analyzed. Verification of the phase and reconstruction of the haplotype was made possible by analyzing DNA samples of relatives. Calculation of the age of the haplotype was performed as described before with the assumption that a generation equals 20 years[15]. An estimation of the marker frequency in the general population of the first recombinant markers on both sides of the *LMNA* gene in the probands was made by analyzing these markers in 96 unrelated control individuals. Markers D1S305 and D1S2624 were used for this purpose.
To find common ancestors in these different families, we also performed genealogical searches using community registries and official records of births, marriages and deaths.

Linkage analysis

Linkage analysis was performed in the families D, E, G, I, L, M, P and Q. For this purpose we used the linkage program GRONLOD.16 The model assumptions we used are described in the Supplemental Materials.

Nuclear morphology of *LMNA* p.(Arg331Gln) fibroblasts

For detailed information about the immunofluorescence staining, see Supplemental Materials. Fibroblasts obtained from a skin biopsy from a patient carrying the *LMNA* p.(Arg331Gln) variant were stained with the antibody JoL2 for detection of Lamin A/C and then counterstained using DAPI. Structural abnormalities of the nuclei were scored based on abnormalities of nuclear shape and according to the following categories: normal, presence of herniations (blebs), honeycomb structures and/or presence of donut-like nuclear invaginations. They were also compared to nuclear morphology data available from control dermal fibroblast cultures.

Electronic microscopic imaging of the nucleus

See Supplemental Materials for a detailed explanation of the electron microscopy (EM) imaging. Two myocardial biopsies of patients carrying the *LMNA* p.(Arg331Gln) mutation were fixed with Karnovsky's Fixative, embedded in Epon, and cut into 70 nm sections. They were then viewed with a FEI Tecnai T12 Electron Microscope.

Maximal force development of the sarcomeres

For a detailed description, see Supplemental Materials. Single cardiomyocytes from patients carrying *LMNA* p.(Arg331Gln) and from control hearts were membrane-

permeabilized and glued between a force transducer and piezo motor. Force development was induced by transferring the cell to solutions of calcium with different concentrations (ranging from physiologic concentrations to a saturating calcium concentration). Force development was recorded with the force transducer. In a later stage, maximal force generation was corrected for myofibril density, measured on EM images.

Statistical analysis

Descriptive statistics are reported as frequency or mean ± standard deviation. We used Kaplan-Meier survival to determine the cumulative event-free survival in *LMNA* p.(Arg331Gln) carriers. We used the Log-rank test to compare the outcomes for *LMNA* p.(Arg331Gln) carriers to those of other pathogenic *LMNA* mutation carriers. For the Kaplan-Meier survival analyses, we used MedCalc Statistical Software version 17.1 (MedCalc Software bvba, Ostend, Belgium). An independent two-sided t-test was used to compare the nuclear irregularities. The data was analyzed with the Statistical Package for Social Sciences (SPSS software version 23.0 (IBM Corp., Armonk, NY, USA)). Force development between groups was compared by Student's t-test after normal distribution was confirmed by Shapiro-Wilk normality test. Statistical analysis on force development was performed by GraphPad Prism 5 software. Data of the force measurements and myofibril density are shown as mean ± standard error of the mean. A p-value <0.05 was considered to represent a significant difference between groups.

Results

Mutation analysis

The NGS cardiomyopathy panel was performed in 22 probands. Fourteen additional variants were found in 13 probands with the targeted cardiomyopathy panel, of which one was labelled as 'pathogenic' and the others as 'variant of unknown significance' (Supplementary Table 1). Screening of the major lipodystrophy genes (*CAV1, PLIN1, PPARG, AKT2*) with whole exome sequencing (WES) was negative for the proband A-III. Analyses of cardiomyopathy related genes screened with WES identified no additional mutation (a list of the screened genes is available on request).

The *LMNA* p.(Arg331Gln) variant was found twice in the Exome Aggregation Consortium dataset (allele frequency 0.0015%) and four times in the GoNL database (allele frequency 0.4%)[17,18].

Clinical evaluation

For a complete overview of clinical features in mutation carriers see Table 1 and Supplementary Table 2. Twenty-three probands were identified, of whom 21 presented with cardiac symptoms, one with symptoms of a partial lipodystrophy and one was identified after family screening following sudden cardiac death. Thirty-five family members were identified as carrying the mutation. Sixteen family members were already known to have cardiac symptoms prior to genetic family screening (8 males, mean age of presentation 56 ± 7 years), 18 family members were evaluated for the first time in the course of family screening (9 males, mean age at first clinical examination 47 ± 12 years) and from one family member no cardiologic information was available. In both probands and family members, there was a high incidence of (paroxysmal) atrial fibrillation (52% and 42%, respectively) and atrioventricular conduction delay (61% and 44%, respectively). Ventricular arrhythmias were frequently reported in both groups, although the occurrence seemed to be higher in the proband group (83% vs. 40%). Twenty-two of 23 (96%) probands had structural abnormalities of the myocardium, of which 17 (74%) were classified as DCM. Structural abnormalities were present in only 11 (38%) family members, of which four were classified as DCM. The overall mean age at the diagnosis of DCM was 50 ± 15 years. End stage heart failure was seen in six carriers, of whom five received an HTx. Two patients died of heart failure, of whom one received an LVAD while awaiting a HTx. Two patients had an aborted cardiac arrest and two appropriate ICD therapy shocks were administered in total.

Explanted hearts of two patients after HTx showed extensive involvement of the right ventricle. The right ventricle even seemed to be predominantly involved in all three members of family Q. One family member fulfilled the revised Task Force criteria for borderline arrhythmogenic right ventricular cardiomyopathy (ARVC): he had one major criterion (regional akinesia and an end diastolic volume over 110 ml/m2 measured by MRI) and one minor criterion (non-sustained ventricular tachycardia observed during X-ECG). In the other two family members, a widened right ventricle was observed (MRI; end diastolic volume of 103 and 109 ml/m2) with a inhomogeneous contraction pattern. In one of them there was also focal bulging of the right ventricle. The left ventricle function was normal in all three subjects.

In family A the mutation seemed to segregate only with a partial lipodystrophy (Supplementary Figure 1). In both the proband (A-III) and her mother (A-II) there was loss of adipose tissue around the extremities and accumulation of adipose tissue on the abdomen, neck and face. Other manifestations were hypertension and hypertriglyceridemia. The proband (A-III) in addition had acanthosis nigricans. The mother (A-II) additionaly suffered from hepatic steatosis and diabetes mellitus type 2. The maternal grandmother had diabetes type 2 and was told to have the same physical

appearance as A-II and to have unspecified cardiac problems. She died suddenly aged 72 years. During follow up, a dilated left ventricle (end diastolic dimension 62 mm) with systolic dysfunction (ejection fraction of 46%) was observed in the proband (A-III, Supplementary Figure 1).

Table 1. Summary of Characteristics of the Probands & Family Members Carrying the *LMNA* p.(Arg331Gln) Mutation

Characteristics	Probands (N=23)	Family members (N=35)
Age presentation/evaluation, yrs (n = 23 & n = 29)*	47 ± (14)	51 ± (12)
Male	17 (74)	18 (51)
Symptoms		
Palpitations (n = 16 & n = 20) *	8 (50)	6 (30)
Syncope (n = 17 & n = 22) *	4 (24)	2 (9)
NYHA class ≥3 (n = 16 & n = 28) *	5 (31)	2 (7)
AV block (n = 18 & n = 25)*		
1st degree	8 (44)	10 (40)
2nd degree	3 (17)	1 (4)
Intraventricular conduction delay (n = 20 & n = 28)*		
LBBB	11 (55)	5 (18)
RBBB	3 (15)	1 (4)
Aspecific		4 (14)
Supraventricular arrhythmias (n = 23 & n = 31)*		
Paroxysmal atrial tachycardia	4 (17)	3 (10)
(Paroxysmal) atrial fibrillation	12 (52)	13 (42)
Ventricular arrhythmias (n = 23 & n = 30)*		
>500 PVCs		2 (7)
NSVT	13 (57)	9 (30)
VT/VF	6 (26)	1 (3)
PM and/or ICD implantation (n = 22 & n = 34)*	17 (77)	6 (18)
Cardiomyopathy (n = 23 & n = 29)*		
DCM	17 (74)	4 (14)
Mild DCM	5 (22)	6 (21)
Other structural abnormalities		1 (3)
HTx or end stage heart failure (n = 23 & n = 30)*	5 (22)	1 (3)

Table 1. Summary of Characteristics of the Probands & Family Members Carrying the *LMNA* p.(Arg331Gln) Mutation *(continued)*

Characteristics	Probands (N=23)		Family members (N=35)	
Comorbidity				
Hypertension (n = 23 & n = 29)*	4	(17)	8	(28)
Coronary artery disease (n = 23 & n = 28)*	1	(4)	3	(11)
Diabetes Mellitus type 2 (n = 23 & n = 27)*	2	(9)	1	(4)
Dyslipidemia (n = 23 & n = 27)*	1	(4)	3	(11)
Medication (n = 23 & n = 26)*				
Anti-arrhythmics	22	(96)	11	(42)
ACE inhibitor or ARB	22	(96)	6	(23)
Diuretics	14	(61)	7	(27)

Values are mean ± (standard deviation) or n (%) *number with available data (probands & family members). Data is summary of the data collected to last follow-up.ACE = Angiotensin-converting enzyme, ARB = Angiotensin receptor blocker; AV = Atrioventricular; DCM = Dilated Cardiomyopathy; HTx = Heart transplantation; ICD = Implantable cardioverter defibrillator; LBBB = Left bundle branch block; (NS)VT = (Non sustained) ventricular tachycardia; NYHA = New York Heart Association; PM = Pacemaker; PVCs = Premature ventricular beats; RBBB = Right bundle branch block; VT/VF = Ventricular tachycardia / Ventricular fibrillation.

Time-to-event analysis

Thirteen *LMNA* p.(Arg331Gln) mutation carriers reached the composite endpoint: appropriate ICD therapy, resuscitation, HTx/LVAD or death. Median survival, i.e. staying free of the composite endpoint, for the p.(Arg331Gln) group was 71 years (CI 95% = 58 to 84 years), which is in contrast to 57 years (CI 95% 53 to 61 years) for carriers of other *LMNA* mutations. Compared to other *LMNA* mutation carriers (both grouped and carriers of only missense mutations) the composite event occurred significantly later in the *LMNA* p.(Arg331Gln) mutation carriers (log-rank; $p<0.001$) (Figure 1). Information on type of *LMNA* mutation and number of carriers is given in Supplementary Table 3. No significant differences were found regarding sex or proband status, comorbidity or use of medication between the groups (Supplementary Table 4).

Haplotype and genealogy

Haplotype analysis was performed in 15 probands and nine family members. A shared haplotype of at least three markers was found covering a 1.00 Mb region surrounding *LMNA* in all the 15 probands (Supplementary Table 5). We calculated that the age of the haplotype containing the mutation is between 340 and 760 years old.

Through genealogical research, we found common ancestors in six families. We could genealogically link family A to family E six generations ago, family J to family M six

generations ago (Supplementary Figure 1 and Supplementary Table 5), and family U to family S four generations ago (pedigrees not shown).

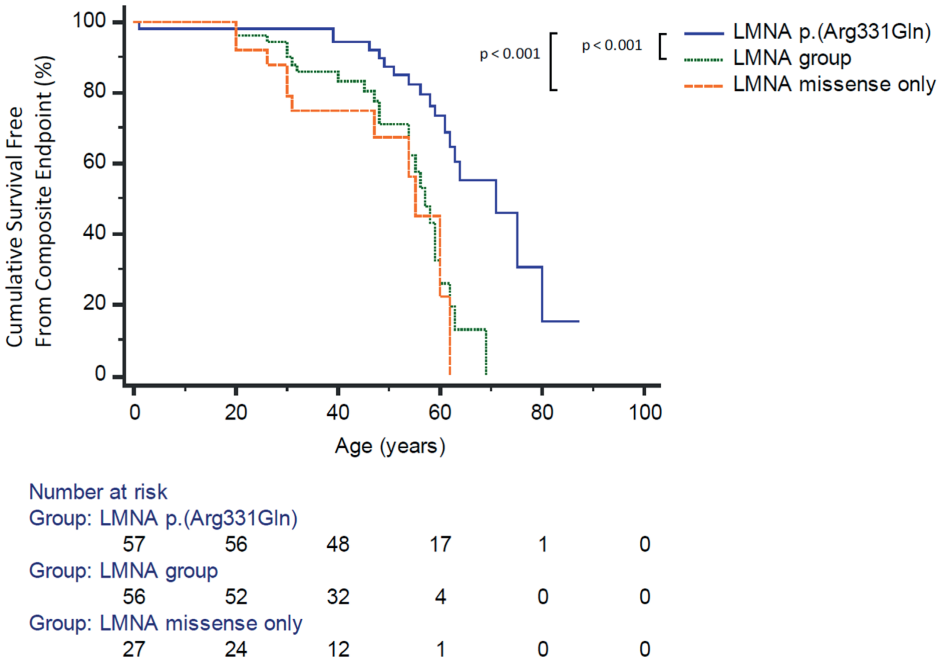

Figure 1. Kaplan-Meier survival analysis

Composite Endpoint = appropriate ICD treatment, resuscitation, HTx/LVAD, death. LVAD = left ventricular assist device. *LMNA* p.(Arg331Gln) carriers had a significantly better outcome compared to the "*LMNA* group", which was composed of carriers of different types of *LMNA* mutations. The outcome was also compared to a subgroup of the "*LMNA* group", which consisted of only *LMNA* missense mutations carriers, called the "*LMNA* missense only"group.

Linkage analysis

A combined LOD score of 3.46 was found with linkage analysis in the families D, E, G, I, L, M, P and Q (Supplemental Materials). This adds to the likelihood that *LMNA* p.(Arg331Gln) is linked to the disease. Functional analysis should further substantiate the hypothesis that the observed mutation is the causal one. In family F the segregation was not conclusive. The cardiac phenotype of the *LMNA* p.(Arg331Gln) negative mother, who had a reduced left ventricular ejection fraction (46%) and frequent ventricular ectopic beats (±18000/24 hours), could also be explained by the fact that she is a carrier of the pathogenic *SCN5A* p.(Gly1319Val) mutation (F-I-1, Supplementary Figure 1)[19], as there are more cases described in which *SCN5A* mutations are associated with DCM[20,21]. The

family history reported that the father of the proband, an obligate carrier of the *LMNA* p.(Arg331Gln) mutation, died at the age of 56 years and that the paternal grandfather had a pacemaker.

Nuclear morphology of *LMNA* p.(Arg331Gln) fibroblasts

Nuclear morphology was analyzed using immunohistochemical staining for lamin A/C in fibroblasts of an *LMNA* p.(Arg331Gln) carrier (proband I-II-1). Next generation cardiomyopathy panel analysis revealed no additional mutations in this patient. The morphology of 496 nuclei were analyzed. An irregular structure was observed in 22.0 ± 6.4% of the p.(Arg331Gln) nuclei, with a honeycomb-like nuclear structure the most frequently observed irregularity (13.6 ± 8.3%; Figure 2). Nuclear blebbing and donut-shaped nuclei were observed in 5.8 ± 3.7% and 2.0 ± 1.4% of the p.(Arg331Gln) fibroblasts, respectively. The findings are consistent with abnormalities of the nuclear membrane in other pathogenic *LMNA* mutations[22]. In contrast, the eight control fibroblast cultures displayed fewer nuclear irregularities, 5.9 ± 1.4% ($p < 0.01$), of which 0.9 ± 1.1% were honeycomb structures ($p < 0.01$), 2.1 ± 1.8% ($p = 0.04$) nuclear blebbing and 1.4 ± 0.7% (no significant difference) donut-shaped nuclei (data not shown).

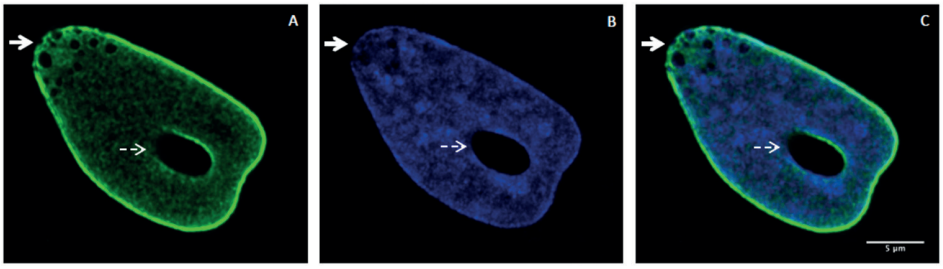

Figure 2. Nuclear envelope immunostainings of skin fibroblasts of an *LMNA* p.(Arg331Gln) carrier

A: Lamin A/C staining with antibody JoL2. **B:** DAPI staining. **C:** Lamin A/C staining and DAPI staining merged. Broken white arrow indicates donut-like nuclear invaginations. Continuous white arrow indicates honeycomb-like nuclear structure.

Electron microscopic imaging of the nucleus

EM of myocardial biopsies of two patients demonstrated the irregular and convoluted shapes of the enlarged nuclei of the cardiomyocytes (Figure 3). In patient P-III-2 (family-member), carrier of additional variant of unknown significance in the gene encoding desmoplakin (DSP) (DSP p.(Lys2706Met)), blebs of the nuclear membrane into the cytoplasm were observed (Figure 3B). In proband B, who carried two additional variants of unknown significance (*LMNA* p.(Arg156Leu) and *TTN* p.(Phe9717Serfs*23)), a

discontinuous layer of heterochromatin of the inner nuclear membrane was observed in several areas of the nuclei. In this patient some small indications of blebs were observed, but larger ones were not evident. Since Lamin A and C play a role in the structural stability of the nuclear membrane, the ultrastructural defects of the nuclear membrane described above are often seen in conjunction with *LMNA* mutations[23,24].

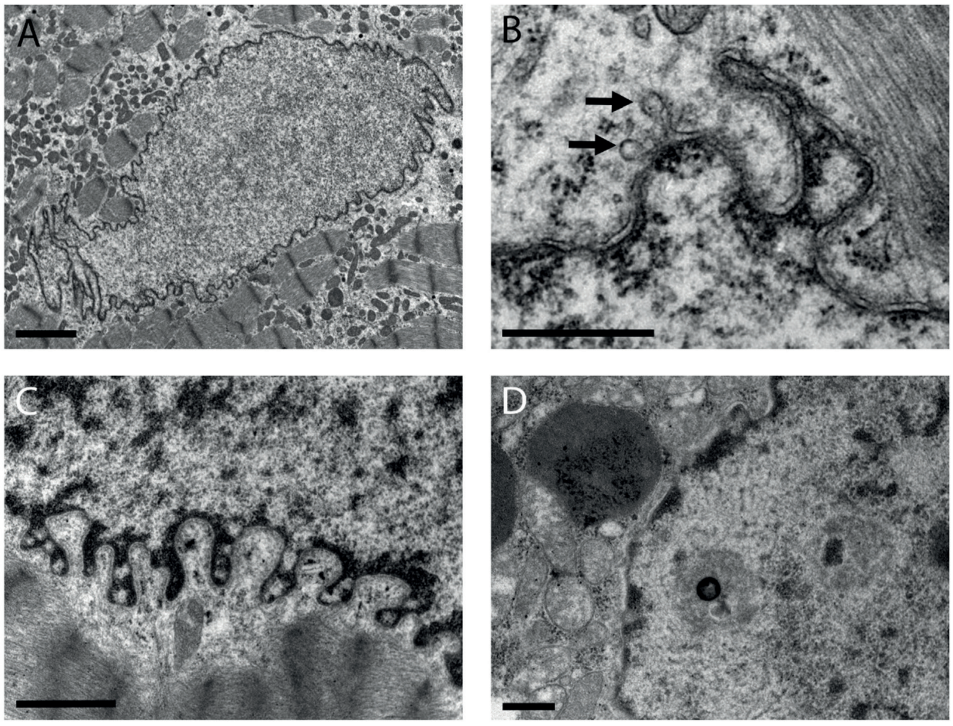

Figure 3. Electron microscopy of myocardium of Patients P-III-2 and B-III-1

A and B: Patient from family P. **A:** Nucleus of cardiomyocyte with convoluted shape. Bar = 2 µm. **B:** detail of nuclear membrane with small blebs of the nuclear membrane into the cytoplasm (arrows). Bar = 500 nm. **C and D:** Proband from family B. **C:** irregular shape of the nuclear membrane. Bar = 1 µm. **D:** detail of nuclear membrane showing a discontinuous layer of chromatin of the nuclear membrane, possible enlarged nuclear pores. Bar = 500 nm.

Maximal force development of the sarcomeres

In Figure 4A a cardiomyocyte of a control heart and in Figure 4B a cardiomyocyte of a patient with the *LMNA* p.(Arg331Gln) variant is visualized at sarcomere length 2.2 µm. Cardiac tissue of three carriers (B-II-1, N and P-III-2) was used for this analysis. B-II-1 carried two additional variants and P-III-2 carried one additional variant of unknown significance (Supplementary table 2). After transfer of the cell to a solution containing

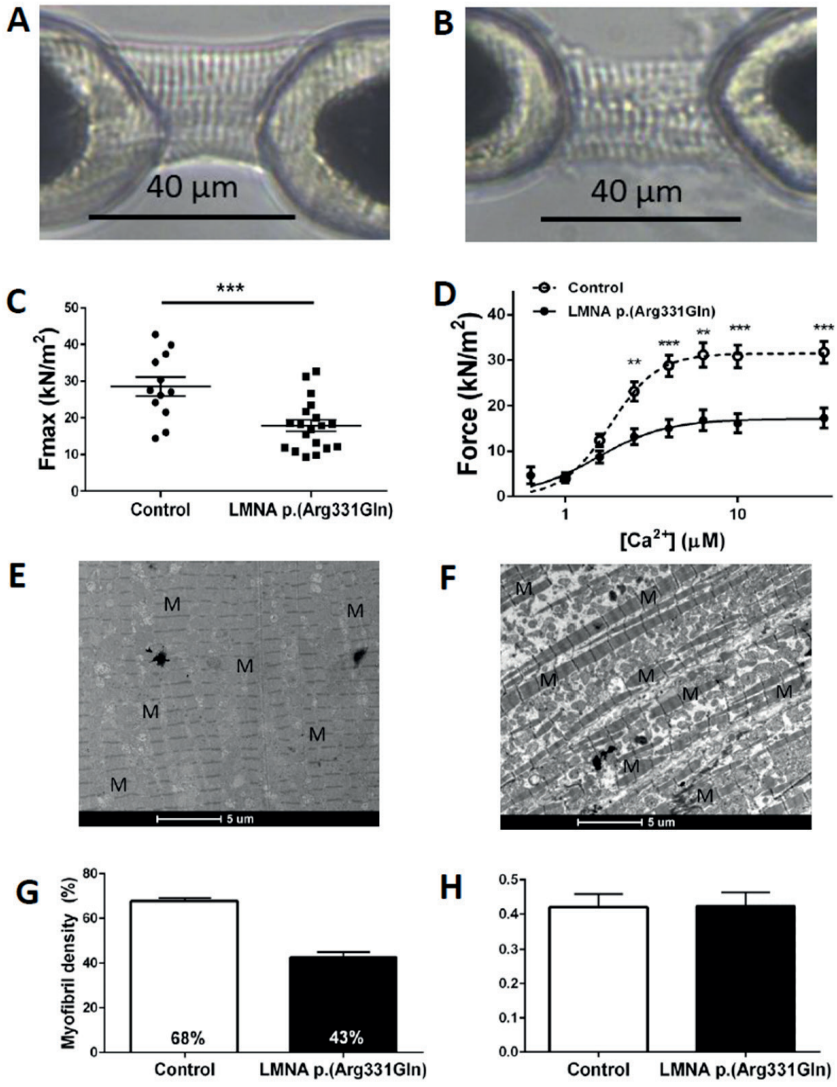

Figure 4. Maximal force development of the sarcomeres

A and B: Mechanical isolated cardiomyocyte of a control heart (**A**) and a cardiomyocytes of a patient with the *LMNA* p.(Arg331Gln) mutation (**B**) glued between a force transducer and piezo motor. **C:** Patients with the *LMNA* p.(Arg331Gln) mutation (N = 3, n = 19) showed a significantly decreased maximal force development compared with controls (N = 2, n = 12) (p = 0.002). **D:** Force development was significantly lower in p.(Arg331Gln) samples (N = 3, n = 11) compared to controls (N = 2, n = 9) over a range of submaximal (physiological) calcium concentrations. **E, F and G**: Myofibril density was lower (**F**) in the *LMNA* p.(Arg331Gln) patients (N = 3 (G)) compared to (**E**) control hearts (N=2 (**G**)). **H:** Maximal force development corrected for myofibril density was similar in the *LMNA* p.(Arg331Gln) patients (N = 3, n = 19) compared to the control hearts (N = 2, n = 12). For each sample, at least four electron microscopy images at a magnification of 2250x were used to calculate average the myofibril density. N= number of patients, n=number of cardiomyocytes.

an activating concentration of calcium, the cell developed force, which was recorded by the force transducer. Patients with the *LMNA* p.(Arg331Gln) mutation showed a significantly decreased maximal force development (17.9 kN/m2) compared with controls (28.5 kN/m2) (p = 0.002, Figure 4C). This indicates an effect of the variant through impairment of cardiomyocyte contractility. As shown in Figure 4D, force development was significantly lower in p.(Arg331Gln) variant samples compared to controls over a range of submaximal (physiological) calcium concentrations. In some cases of hypertrophic cardiomyopathy a reduction of myofibril density underlies the lower force generating capacity[25]. We hypothesized that this could also be the case for the *LMNA* p.(Arg331Gln) variant. Myofibril density was calculated as a percentage of total cardiomyocyte area by EM, and myofibril density was found to be lower in the hearts of *LMNA* p.(Arg331Gln) patients (Figure 4F) when compared to control hearts (Figure 4E). Myofibril density was 43% in the *LMNA* p.(Arg331Gln) patient hearts compared to 68% in control hearts (Figure 4G). Maximal force development corrected for myofibril density was similar in the *LMNA* p.(Arg331Gln) patients compared with control hearts (Figure 4H). This indicates that the decreased force generation observed in the p.(Arg331Gln) mutation in *LMNA* is probably due to the reduced myofibril density.

Discussion

Interpretation of missense mutations is especially challenging when a variant is also present in a control population, the situation we encountered here for the *LMNA* p.(Arg331Gln)) variant. Although we are not the first to describe the *LMNA* p.(Arg331Gln) variant, we were able to collect the largest cohort of carriers to date. In a previous report this variant was found in a patient who was compound heterozygous (carrier of the *LMNA* p.(Glu347Lys)). In addition, another variant at the same position, *LMNA* p.(Arg331Pro), was described to be associated with DCM, conduction delay and limb-girdle muscular dystrophy[26]. In another report the parents of the proband were not screened for the mutation and were seemingly unaffected (only the father had atrial fibrillation)[27]. Extensive evaluation of clinical, segregation and functional data helped us to classify this mutation as truly pathogenic and we decided to communicate this as such to the carriers of this mutation. Moreover, its associated phenotype is consistent with that described in carriers of other *LMNA* mutations but milder in terms of significant clinical events (malignant arrhythmias, end stage heart failure, or death).

The phenotype in our cohort is characterized by a high incidence of (paroxysmal) atrial fibrillation ((p)AF) and atrioventricular conduction delay in both probands and family members ((p)AF, 52% and 42%: AV-block, 61% and 44%). These findings are consistent with the clinical findings of two studies of 269 and 299 *LMNA* mutation carriers[4,6*]. These

LMNA mutation carriers demonstrated a high prevalence of atrial tachyarrhythmia (36%) and conduction disease (47%)[6]. More specifically, Fatkin et al described four missense mutations in the rod domain, where the p.Arg331Gln variant is also situated, with a phenotype (AV-conduction delay, atrial fibrillation, sinus bradycardia and DCM) similar to that seen in our cohort[28]. However, malignant ventricular arrhythmias (appropriate ICD therapy, SCD, OHCA, ventricle fibrillation) did not seem to occur as often (12%) in our cohort compared to *LMNA* patient series described in literature, where malignant ventricular arrhythmias were observed in 24% to 28% of the cases[6,29]. The milder phenotype in our carriers is corroborated by the relatively infrequent occurrence of appropriate shock and ATP therapy (only two shocks and three ATP therapies in 86 patient years). In both cases of ICD shock, there were additional factors that could have played a role (poor LV systolic function and evidence of an old myocardial infarction). This is in contrast to the observation in patients with other *LMNA* mutations where 28% to 42% of the carriers seemed to benefit (appropriate therapy) from ICD implantation[6,8]. The diagnosis of DCM in our cohort was made relatively late in life (50 ± 15 years), compared to that of the group DCM patients carrying other pathogenic *LMNA* mutations, for whom an age of onset of 40 ± 10 years is described[29]. Structural abnormalities were only apparent in eleven (38%) family members. However, 65% of the family members had electric disturbances of the heart (evidence of sinus node dysfunction, cardiac (AV) conduction delay, atrial or ventricular arrhythmias, with no structural abnormalities of the heart (yet)). In *LMNA* mutations, it is a well-known phenomenon that electric abnormalities, like conduction delay and arrhythmias, often precede the structural abnormalities[4,28] . Regular follow-up is warranted because these initial electric abnormalities could be the first signs of structural abnormalities, which could be followed by an impaired function and LV dilatation.

One notable aspect of our study is the pleiotropic effect of the *LMNA* p.(Arg331Gln) mutation, which is demonstrated by the differences in phenotypes between the families. This is most striking in family A where the mutation seemed to segregate with a partial lipodystrophy (PLD) phenotype. When PLD is associated with *LMNA* mutations, the disease is also referred to as FLDP2 (familial partial lipodystrophy type 2), which is an autosomal dominant disease that mostly results from missense mutations in the C-terminal region of the *LMNA* gene and is characterized by progressive abnormal subcutaneous adipose distribution[30]. However mutations in the N-terminal head and alpha-helical rod domain in which the FLDP2 is accompanied by cardiomyopathy and conduction disorders have also been described[31]. The apparent absence of this lipodystrophic phenotype in the other families suggests there is another possible genetic cause. However, screening of other major lipodystrophy genes was normal. Other results of the pleiotropic effects of this mutation are the findings of the apparent

solely RV involvement in family Q. Recently, another *LMNA* mutation (p.(Leu140_Ala146dup)) was described as associated with both ARVC and DCM[32]. In two other studies, genetic screening in patients with ARVC revealed five missense mutations and one nonsense mutation in *LMNA* in the absence of mutations in the desmosomal genes[33,34]. Although DCM was the predominant form of cardiomyopathy in our cohort, RV involvement was seen in 57% of the patients with DCM and the available pathology reports in two probands describe extensive right ventricle involvement. This suggests that *LMNA* related disease may mimic ARVC. The heterogenous phenotype might be influenced by additional genetic factors (Supplementary Table 2), yet this series of patients is too small to systematically evaluate this.

Marker analysis showed a common haplotype of 1 Mb, suggesting a founder mutation. Slippage during replication of DNA in one of the ancestors could explain the difference in length of marker D1S1153 found in the two groups.

Although most of the families were small, a dominant autosomal inheritance pattern could be observed. The calculated combined LOD score was well over 3, an additional observation suggesting pathogenicity of this variant. A limitation of the segregation analysis is the fact that we counted a subject as affected when he or she displayed one of the phenotypes commonly observed with *LMNA* mutations. As described earlier, the phenotype can be highly variable and some of the phenotypes, e.g. AF and conduction disease, are also found in relatively high frequencies in the general population in absence of *LMNA* mutations. To take this into account we calculated the LOD scores with a phenocopy frequency (e.g. AF 10%, conduction disease 10%) higher than expected for the general population (see Supplemental Materials). Still, that resulted in a LOD score of more than 3. Non-segregation was observed possibly once and has been described before in a large *LMNA* family[35]. In family F the cardiac phenotype of the mother (F-I-1) could be explained by a pathogenic *SCN5A* mutation, as it is recognized that *SCNA5A* mutations can also cause DCM[20]. The same *SCN5A* mutation was also found in our laboratory in two unrelated patients with cardiomyopathy, while screening of 53 or 55 other cardiomyopathy-related genes revealed no additional mutations in those subjects (unpublished data).

Lamins A and C are important components of the nuclear envelope and, when mutated, abnormalities of the nuclear envelope can be observed.[22] Irregular nuclear structures were significantly more frequently observed in fibroblasts of a *LMNA* p.(Arg331Gln) carrier. The nuclear abnormalities observed in the p.(Arg331Gln) fibroblasts are in line with the nuclear irregularities observed in fibroblasts of other established pathogenic mutations in LMNA, thereby supporting its pathogenicity[22,36,37,38.]

Like the abnormal nuclear structures in the fibroblasts observed using immunofluorescence, ultrastructural investigation on diseased cells in cardiac tissue with EM also support pathogenicity. The convoluted shapes of the nuclei, blebs, discontinuous layer of heterochromatin and possible enlarged nuclear pores are features commonly seen in other *LMNA* mutations[23,24,39] It should be kept in mind that such structural defects can also be found in DCM patients without *LMNA* mutations[40].
Apart from their function in nuclear stability, it has been suggested that lamin proteins are important for the structural integrity of the whole cell through interactions between nuclear lamina, the cytoskeleton and the extracellular matrix[41,42] The lamin A/C coil 2B domain in which the p.(Arg331Gln) mutation is located is important for homodimerization of lamin proteins. Gangemi et al. indicated that the p.(Arg331Gln) mutation might affect lamina stability, because it has been predicted to impair dimerization of the lamin proteins due to loss of salt-bridge interactions[43]. This might explain the detrimental effect on the heart since correct assembly of dimers is essential for protein function. In addition, it is known that myofilaments in cardiomyocytes create nuclear deformation in the plane parallel to the myofilaments during contraction[42]. Therefore, the continuous mechanical stress during contractions in cardiomyocytes can have pathological effects on nuclear structure over time in patients with the p.(Arg331Gln) mutation. Our study supports this possibility by showing impairment of nuclear architecture and decreased myofibril density in patients with the p.(Arg331Gln) mutation causing a reduction in cardiomyocyte force development.

Limitations

The observational design of this study is prone to introduction of survival bias. Initially, genetic testing focused on the most severely affected cases and currently patients with a less severe phenotype are more easily referred for genetic testing. However, this study includes all patients that were identified since the start of *LMNA* screening in 2001 and therefore is likely to reflect both sides of the spectrum. The combined LOD score of more than 3 does not mean significant linkage due to a possible selection bias in the families selected. It should be considered a strong indication that the mutation segregates with the observed phenotypes.

Conclusion

Genetic and segregation data support the pathogenic effects of *LMNA* p.(Arg331Gln). Electron microscopy and immunofluorescence showed an effect on nuclear architecture. In addition, the *LMNA* p.(Arg331Gln) mutation causes decreased myofibril density resulting in reduced force development at saturating and physiological calcium concentrations. The clinical phenotype related to the *LMNA* p.(Arg331Gln) founder mutation is generally characterized by a phenotype (consisting of cardiac conduction delay, (atrial) arrhythmias, and dilated cardiomyopathy with a later onset and more favorable prognosis compared to other pathogenic *LMNA* mutations. Further research is needed to elucidate the role of other contributing factors leading to the clinical variability.

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Disclosures

None

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Supplemental methods

Clinical evaluation:

Sinus node dysfunction was defined as sinus bradycardia of less than 50 beats per minute on a resting ECG, chronotropic incompetence (inadequate increase of heart rate (≤ 80%) of expected value, during X-ECG) and pauses of more than three seconds observed with Holter monitoring during sinus rhythm or two seconds when atrial fibrillation was the underlying rhythm. Dilated cardiomyopathy (DCM) was defined by 1) diminished left ventricular contractile function (left ventricular ejection fraction <45% and or left ventricular shortening <25%) and 2) ventricular dilatation (left ventricular end-diastolic dimensions >95th percentile indexed for body surface area, age and sex in case of MRI).[1–3]. If only one of the two criteria was fulfilled (either left ventricular dilatation or dysfunction), the patient was labeled as having 'mild DCM'. If bodyweight or end diastolic diameter was unavailable, we still recorded a diagnosis of DCM if this was stated in the clinical information. A diagnosis of borderline arrhythmogenic right ventricular cardiomyopathy
(ARVC) was made when one major criterion and one minor criterion or three minor criteria from different groups were fulfilled according the modified Task Force criteria.[4]

Linkage analysis 'GRONDLOD':

The aim of linkage analysis is to test if a genetic variant cosegregates in a genome wide significant way with observed phenotypes in affected families. *LMNA* mutations are associated with a clinically variable phenotype. The phenotypes observed are, however, not exclusively found in the presence of *LMNA* mutations and some of these phenotypes have a relatively frequent occurrence in the general population (increasing with age), atrial fibrillation and conduction disease for example. To take account for possible phenocopies and age-related penetrance, we used the linkage program GRONDLOD because this program has the advantage of easily defining genetic model assumptions with complex phenotype-genotype relations.[5] Families D, E, G, I, L, M, P and Q were used to calculate LOD scores at zero recombination frequency (Supplementary figure 1). Linkage analysis cannot prove that genetic variants are causally implicated in a disease, only that the co-segregation of a variant with a phenotype is more likely if the observed variant is associated with an assumed causative locus than when it is unlinked.
The following model assumptions were used for the computation:
We defined phenotypes as follows (abbreviations); unknown, normal, supraventricular tachycardia (SVT), atrial fibrillation (AF), conduction disease (CD), dilated cardiomyopathy (DCM), dilated cardiomyopathy with conduction disease (CD_DCM), cardiomyopathy

with atrial fibrillation and conduction disease (AF_CD_CM) and ventricular arrhythmias (VA). For family Q a phenotype cardiomyopathy unspecified' (CM_unsp) was used because although there were apparent structural abnormalities in all three family members, only one fulfilled the diagnosis for borderline ARVC. Because no definite diagnosis of a cardiomyopathy could be made, we allowed for a higher frequency of phenocopies (10%).

The disease gene is considered to be a rare variant and the allele frequency was set to 0.001 and the normal variant to 0.999.

The probabilities for a certain phenotype given an genotype were as follows:
Explanation: the first number after the curved bracket is the disease locus, the second and third numbers describe the autosomal marker phenotype and the disease phenotype follows between the double quotes. The last number reflects the assumed probability of the genotype conditional on the disease status genotype.


```
phen_gen(1,1,1,"SVT",0.3) // allows for 30% phenocopies
phen_gen(1,1,2,"SVT",0.5) // 50% penetrance for carriers
phen_gen(1,2,2,"SVT",1)
phen_gen(1,1,1,"AF",0.1) // allows for 10% phenocopies
phen_gen(1,1,2,"AF",0.5) // 50% penetrance for carriers
phen_gen(1,2,2,"AF",1)
phen_gen(1,1,1,"CD",0.1) // allows for 10% phenocopies
phen_gen(1,1,2,"CD",0.5) // 50% penetrance for carriers
phen_gen(1,2,2,"CD",1)
phen_gen(1,1,1,"AF_CD",0.05) // allows for 5% phenocopies
phen_gen(1,1,2,"AF_CD",0.4) // 40% penetrance for carriers
phen_gen(1,2,2,"AF_CD",1)
phen_gen(1,1,1,"DCM",0.005) // allows for 0.5% phenocopies
phen_gen(1,1,2,"DCM",0.5) // 50% penetrance for carriers
phen_gen(1,2,2,"DCM",1)
phen_gen(1,1,1,"CD_DCM",0.001) // allows for 0.1% phenocopies
phen_gen(1,1,2,"CD_DCM",0.4) // 40% penetrance for carriers
phen_gen(1,2,2,"CD_DCM",1)
phen_gen(1,1,1,"AF_CD_CM",0.001) // allows for 0.1% phenocopies
phen_gen(1,1,2,"AF_CD_CM",0.4) // 40% penetrance for carriers
phen_gen(1,2,2,"AF_CD_CM",1)
phen_gen(1,1,1,"VA",0.2) // allows for 20% phenocopies
```

phen_gen(1,1,2,"VA",0.5) // 50% penetrance for carriers
phen_gen(1,2,2,"VA",1)
phen_gen(1,1,1,"CM_unsp",0.1) // allows for 10% phenocopies
phen_gen(1,1,2,"CM_unsp",0.5) // 50% penetrance for carriers
phen_gen(1,1,2,"CM_unsp",1)

Nuclear morphology of *LMNA* p.(Arg331Gln) fibroblasts:

Fibroblasts from a skin biopsy taken from one patient (proband I-II-1) with the *LMNA* p.(Arg331Gln) were cultured for immunostaining. Early passage cells (p3-5) were seeded at a low density and were allowed to attach for two days before fixation. Detection of lamin A/C was performed using antibody JoL2 (kindly provided by Dr. C. Hutchison, Durham University, UK) as described previously.[6] The nuclei were counterstained using DAPI. Imaging was performed by means of an inverted confocal microscope (Leica SPE) mounted on a DMI4000 inverted microscope. Per fibroblast culture, the morphology of at least 5x100 nuclei was scored by two independent researchers. Nuclear morphology scores were based on abnormalities of nuclear shape and irregular immunostaining for lamin A/C. Nuclei were scored according to the following categories: normal; presence of herniations (blebs); and/or honeycomb structures (visualized by immunolabelling with JoL2); presence of donut-like nuclear invaginations.

Electronic microscopic imaging of the nucleus:

Myocardial biopsies of the apex of the heart of two patients (family-member P-III-2 and proband B) obtained during implantation of a LVAD were studied with electron microscopy (EM). Myocardium was fixed in Karnovsky's Fixative and embedded in Epon, and 70 nm sections were cut. The sections were mounted onto formvar-coated copper grids (Stork Veco, Eerbeek, the Netherlands) and stained with a 5% solution of uranyl acetate, followed by Reynold's lead citrate. Sections were viewed with a FEI Tecnai T12 Transmission Electron Microscope (FEI, Hillsboro, Oregon, USA).

Maximal force development of the sarcomeres:

Force measurements in membrane-permeabilized single cardiomyocytes and EM images were acquired from three patients with the *LMNA* p.(Arg331Gln) mutation. Myocardial biopsies of the apex of the heart were obtained prior to LVAD implantation in two patients. In one patient, explanted left ventricular myocardial tissue was collected during heart transplantation. Left ventricular cardiac tissue of two patients who died of a non-cardiac cause served as controls.

Force development of sarcomeres was measured in single membrane-permeabilized cardiomyocytes mechanically isolated from heart tissue as previously described[7] and corrected for cross sectional area. Mechanically isolated cardiomyocytes were glued between a force transducer and piezo motor and stretched to a sarcomere length of 2.2 µm. Force development was recorded with the force transducer attached to the cell. When force development reached a plateau, the cell was shortened by 30% of its length in order to detach cross-bridges and determine the total force generated. Total force development was calculated by the difference between force at plateau and force at slack length. The cell was then transferred back to a relax solution where it was again shortened by 30% to calculate passive force development. Maximal force development was calculated by subtraction of passive force from total force at a saturating calcium concentration of 31.6 µM. In addition, force development was also measured at a range of submaximal (physiological) calcium concentrations.

Myofibril density:

EM pictures were analyzed at a magnification of 2250x. Myofibril density was calculated as a percentage of cardiomyocyte area by ImageJ software. For each sample 4 or 5 images were used to calculate average myofibril density. Maximal force was corrected for myofibril density by dividing the maximal force generated by individual cardiomyocytes by the average myofibril density of the corresponding sample.

Supplemental tables

Supplementary Table 1. Other Genetic Variants identified

Gene	Position	Protein	Effect	Classification	Description
ANKRD1	c.651+1G>A		Splice site mutation	VUS	No cardiac abnormalities in the family this variant originated from (family M, Supplementary Figure 1)
DSC2	c.942+3A>G		Splice site mutation	VUS / Likely benign	RT-PCR revealed no aberrant splicing of mRNA in proband carrying this additional mutation.
DSP	c.8500C>T	p.(Arg2834Cys)	Missense	VUS	Did not segregate with phenotype "Mild DCM, conduction disease and atrial fibrillation" in the family where this mutation was found (family G, supplementary Figure 1).
DSP	c.8117A>T	p.(Lys2706Met)	Missense	VUS	Found 5x in ExAC. South Asian background; 3/16512 alleles. European (Non-Finnish) background; 2/66626 alleles.
LMNA A/C	c.467G>T	p.(Arg156Leu)	Missense	VUS	
LMNA A/C	c.1879C>T	p.(Arg627Cys)	Missense	VUS	No nuclear envelope abnormalities were found in fibroblasts of the carrier (brother of proband) with only this variation.
LDB3	c.1885G>A	p.(Ala629Thr)	Missense	VUS	
MYH6	c.3809G>A	p.(Arg1270His)	Missense	VUS	Did not segregate with phenotype "sinus node dysfunction"
RYR2	c.8147A>T	p.(Lys2716Ile)	Missense	VUS	Did not segregate with phenotype "atrial fibrillation" in the family where this mutation was found (family G, supplementary Figure 1).
SCN5A	c.3956G>T	p.(Gly1319Val)	Missense	Pathogenic	Associated with Brugada syndrome.[8] Could be responsible for cardiac phenotype in the mother of proband (family F, supplementary Figure 1).
TTN	c.16452delA	p.(Lys5484Leufs*20)	Frameshift	VUS / Likely benign	Resided in isoform novex-3 transcript, minor small cardiac isoform. Low expression in heart therefore possibly "likely benign".

Supplementary Table 1. Other Genetic Variants identified *(continued)*

Gene	Position	Protein	Effect	Classification	Description
TTN	c.29148delC	p.(Phe9717Serfs*23)	Frameshift	VUS	Resided in exon 103 in the I-band. Not incorporated in major cardiac isoform N2B.
TTN	c.61688T>A	p.(Ile20563Asn)	Missense	VUS	
TTN	c.102275G>A	p.(Arg34092His)	Missense	VUS	Found 6x in European (non-Finnish) population (6/66688 alleles). Not incorporated in major cardiac isoform N2B.

ANKRD1 = Ankyrin Domain 1; DSC2 = Desmocollin 2; DSP = Desmoplakin; LDB3 = LIM Domain-Binding 3; ExAC = The Exome Aggregation Consortium; *LMNA* = Lamin; MYH6 = Myosin heavy chain 6; RYR2 = Ryanodine receptor 2; SCN5A = Cardiac sodium channel α subunit ; TTN = Titin; VUS = Variant of unknown significance.

Supplementary Table 2. Individual *LMNA* p.(Arg331Gln) carrier characteristics ordered by pedigree.

Subject	Sex	Indication genetic screening	Presentation (Age, Yrs.)	Follow-up (Yrs.)	CCD/ECG Abnormalities	SVA	VA	LVF* (EF)	LVEDD* Other	RVF* (EF)	RVEDD* Other	Hypertension	Dyslipidemia	DM type 2	CAD	Additional variant(s)	BMI (kg/m^2)	Outcome	Comment
A-II	F		Atypical symptoms (65)	2	SND	No	No	Normal	41 mm	Normal	.	+	+	+	-	-	24,7	PLD	Beta-blocker Diuretic
A-III	F	PLD	Symptoms of PLD (39)	2	No	No	No	46%	62 mm RWMA	.	.	+	+	-	-	-	34,3	PLD Mild DCM	Beta-blocker ATr antagonist Diuretic
B-III-1	M	DCM	Dyspnea on exertion (35)	13	LBBB AV-block (2nd) Low voltage	No	NSVT	20%	60 mm	Impaired	.	-	-	-	-	+	22,8	DCM CRT-D LVAD, HTx	*TTN* c.29148delc *LMNA* c.467G>T PA: Heart weight 400 gram, extensive diffuse fibrosis
B-III-2	F		Screening (39)	0	No	No	No	55%	50 mm	Normal	Normal	-	-	-	-	+	21,5	Asymptomatic	*TTN* c.29148delc
B-III-3	F		Screening (47)	0	LAHB	No	No	Normal	50 mm	Normal	Normal	-	-	-	-	-	26,3	CCD	
C	M	DCM	Palpitations, AF (61)	16	LBBB SND	AF	NSVT	30%	63 mm RWMA	Impaired	Dilated RWMA	-	-	-	-	-	22,6	DCM CRT-P	
D-II-1	F		MI (62)	11	LBBB AV-block (1st)	AF	NSVT	Normal	.	Normal	.	+	+	-	+	+	.	CVA, death (80)	*TTN* c.61688T>A Beta-blocker
D-III-1	M	DCM	AF (46)	13	LBBB AV-block (1st) Low voltage	AF	NSVT	40%	Dilated† RWMA LGE-	26%	.	-	-	-	+	+	22,9	DCM CRT-D	*TTN* c.61688T>A
D-III-2	M		AF (52)	8	IV cond. delay (124 ms) AV-block (1st)	AF	>500 PVCs/ 24h	27%	58 mm RWMA	.	.	+	-	-	-	.	30,6	DCM Recovery LVF LV hypertrophy	Beta-blocker ATr antagonist Diuretic
E-II-1	M	DCM	Syncope, AF (54)	2	LAHB, RBBB SND	AF	NSVT	37%	62 mm LGE+	54%	Dilated	-	-	-	-	+	20,4	DCM PM, ICD	*MYH6* c.3809G>T
E-II-2	M		Screening (55)	0	SND PVCs on ECG	No	No	Normal	54 mm	Normal	Normal	-	-	-	-	-	23,1	CCD	
E-II-3	M		pAF (48)	4	AV-block (1st) Low voltage	pAF	No	61%	51 mm	Normal	.	-	-	-	-	+	27,8	CCD SVA	*MYH6* c.3809G>T Flecainide
F-II-1	M	Suspected ARVC	Fatigue (40)	2	RBBB SND	No	No	49%	59 mm LGE-	40%	TFC 2010+ Pron. trab.	-	-	-	-	+	21,0	Mild DCM / Possible ARVC PM, ICD	*SCN5A* c.3956G>T ACE inhibitor

Supplementary Table 2. Individual *LMNA* p.(Arg331Gln) carrier characteristics ordered by pedigree. *Continued)*

G-II-1	M	DCM	NSVT, AF (53)	8	LBBB AV-block (1st)	AF	NSVT	28%	66 mm	Poor	.	-	-	+	-	+	26,2	DCM CRT-D HTx	RYR2 c.8147A>T DSP c.8500C>T PA: Heart weight 470 gram, interstitial fibrosis
G-II-2	M		LBBB, pAF (56)	7	LBBB AV-block (2ns) Low voltage	pAF	NSVT	30%	54 mm LGE+	Normal	.	+	-	-	-	+	31,9	Mild DCM CRT-D	RYR2 c.8147A>T DSP c.8500C>T
G-II-3	F		Screening (56)	3	LBBB AV-block (1st)	No	No	49%	47 mm LGE-	49%	43 mm	-	-	-	-	+	28,3	CCD	RYR2 c.8147A>T
G-II-4	F		IV cond. delay (60)	4	LBBB AV-block (1st)	pAF	No	50%	53 mm	Normal		+	-	-	-	-	26,3	Mild DCM CCD SVA	Beta-blocker ACE inhibitor Diuretic
G-III-1	M		Screening (36)	0	No	No	NSVT	Normal	45 mm	.	.	-	-	-	-	-	23,6	VA	
H	M	DCM	pAF (64)	7	.	AF	No	26%	58 mm	Normal	.	+	-	-	-	+	24,8	DCM CRT-D Recovery LVF	*TTN* c.102275G>A Beta-blocker ACE inhibitor Diuretic
I-II-1	F	DCM	Atypical chestpain (54)	0	LBBB AV-block (1st)	No	NSVT	40%	58 mm	.	.	-	-	-	-	-	19,4	DCM CRT-D	
I-II-1	F		CAD (60)	9	IV cond. delay (QRS 120 ms) AV-block (1st)	pAF	No	16%	57 mm RWMA LGE+	Normal	Normal	+	-	-	+	-	22,4	DCM CRT-D	Alcohol abuse
I-III-1	M		Screening (32)	2	IV cond. delay (QRS 145 ms)	No	No	Normal	56 mm	Normal	41 mm	-	-	-	-	-	20,7	Mild DCM CCD	Heavy physical exercise
I-III-2	M		Screening (44)	0	No	No	No	Normal	Normal	Normal	.	-	+	-	-	-	.	Asymptomatic	
J-I-1	M	Obligate carrier	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Liver cirrhosis, dead (48)	
J-II-1	M	DCM	Dyspnea on exertion, palpitations (60)	4	LBBB AV-block (1st)	pAF	NSVT	36%	64 mm LGE+	Poor	Dilated	+	-	+	-	-	25,8	DCM CRT-D ATP therapy	
J-II-2	M		Screening (61)	2	RBBB, LAHB AV-block (1st)	No	No	Normal	56 mm	Normal	43 mm	-	-	-	-	-	27,8	Mild DCM	
J-III-1	F		Screening (28)	0	No	No	No	Normal	48 mm	Normal	Normal	-	-	-	-	-	29,4	Asymptomatic	
K	M	DCM	pAF (56)	11	LBBB AV-block (1st) Low voltage	AF	NSVT	31%	60 mm Pron. trab. LGE+	.	LGE+	-	-	-	-	+	30,0	DCM CRT-D	TTN c.16452delA

Supplementary Table 2. Individual *LMNA* p.(Arg331Gln) carrier characteristics ordered by pedigree. (*Continued*)

L-I-1	M	DCM	LBBB (56)	19	LBBB	AF	NSVT	35%	.	.	.	-	-	-	-	-	23,1	DCM CRT-D ATP therapy	
L-I-2	M	Obligate carrier	Family History: -Rhythm disorder (35)	-	Family History: -Bradycardia -PM	.	.	.	.	.	.	.	.	.	.	.	.	Death (62)	
L-I-3	F	Obligate carrier	LBBB	-	LBBB	AF	NSVT	.	.	.	.	.	.	.	.	.	.	CCD PM	
L-II-1	M		Screening (49)	3	AV-block (1st)	No	No	58%	50 mm Pron. trab.	Normal	Normal	-	-	-	-	-	23,5	CCD	
L-II-2	F		Screening (60)	0	No	Runs of PACs	No	64%	50 mm Pron. trab.	Normal	38 mm	-	-	-	-	-	24,4	SVA	
M-I-1	F		Syncope (62)	6	Low voltage PVCs	pAF	>1000 PVCs/ 24H	Normal	50 mm LGE-	Normal	.	-	-	-	-	-	23,5	SVA VA, ICD	Beta-blocker
M-I-2	F		AF (74)	0	SND	AF	NSVT	Normal	52 mm	Normal	Normal	-	-	-	-	-	21,5	Mild DCM VA, ICD SVA	Radiotherapy
M-I-3	F	.	Screening (60)	0	No	Runs of PACs	No	Normal	44 mm	Normal	Normal	-	-	-	-	-	26,4	SVA	
M-I-4	M	Obligate carrier	-	.	-	pAF	.	.	.	.	.	-	-	-	-	-	.	Sepsis, death (63)	Metastasized Esophagus ca. Autopsy: Hypertrophic heart Heart weight 460 gram
M-II-1	M		Screening (36)	2	No	No	NSVT	53%	.	.		-	-	-	-	-	22,3	VA	
M-II-2	F		Screening (43)	2	No	Runs of PACs	No	59%	50 mm	.		-	-	-	-	-	26,1	SVA	
M-II-3	F		Screening (42)	0	Low voltage	No	No	Normal	44 mm	Normal		-	-	-	-	-	29,0	Low voltage	
M-II-4	M		Screening (39)	0	No	No	No	Normal	43 mm	.		-	-	-	-	-	24,0	Asymptomatic	
M-III-1	F	DCM	OHCA (1)	2	Low voltage	No	VF	34%	35 mm LGE+	45%		-	-	-	-	+	14,9	DCM ICD Partial recovery	ANKRD1 c.651+1G>A Beta blocker ACE inhibitor

Supplementary Table 2. Individual *LMNA* p.(Arg331Gln) carrier characteristics ordered by pedigree. (*Continued)*

N	F	DCM	Dyspnea (44)	4	RBBB	Run of PACs	VT	27%	66 mm	Normal		-	-	-	-	-	29,8	DCM CRT-D Appr. ICD shock LVAD, HTx	PA: DCM phenotype, irregular shape of the nuclei, interstitial fibrosis, small area of fibrofatty replacement in epicardial area of the left ventricle
O-I	M	Obligate carrier	AF (61)	-	.	AF	NSVT	.	.	.	.	.	.	.	.	.	-	Death (71)	
O-II	M	CM	Palpitations, NSVT (44)	2	AV-block (1st) Low voltage	Runs of PACs	NSVT	47%	63 mm, Apical thinning, LGE+	Normal	.	+	-	-	-	-	32,8	Mild DCM SVA VA	Beta blocker ATr antagonist
P-II-1	F	Obligate carrier		.	.	.	.	.	.	.	.	.	.	.	.	.	.	Family History: Sudden death (56)	Family history: -PM indication -Heart failure
P-II-2	F		Palpitations, AF (59)	14	IV cond. delay (QRS 126 ms)	AF	VF	38%	64 mm	Impaired	.	+	-	-	+	-	30,6	OHCA, ICD DCM	
P-III-1	M	CM	Angina Pectoris (47)	5	LBBB AV-block (1st) Sinus node dysfunction	Runs of PACs	NSVT	48%	Dilated† NCCM LGE-	46%	TFC+ NCCM	-	-	-	-	+	29,2	Mild DCM / NCCM	*DSP* c.8117A>T Beta blocker ATr antagonist
P-III-2	M		Palpitation, AF (41)	5	SND Low voltage	AF	NSVT	20%	59 mm	Normal	.	-	-	-	-	+	24,9	LVAD Death due to heart failure (46)	*DSP* c.8117A>T PA: Unregularly nuclei, fibrosis, DCM phenotype
Q-II-1	M	Possible ARVC	Screening (41)	11	Incomplete RBBB	Runs of PACs	NSVT	59%	Dilated† LGE+	47%	Focal bulging, Basally dilated	-	-	-	-	-	27,8	Mild DCM	Beta-blocker
Q-II-2	M		Palpitations (45)	14	Incomplete RBBB, AV-block (1st)	No	No	58%	Normal	47%	Inhom. contraction pattern	-	-	-	-	-	25,9	CCD	Beta-blocker
Q-II-3	M		Screening (44)	11	AV-block (1st)	No	NSVT	55%	Dilated† LGE+	49%	TFC+	+	-	-	-	-	27,7	Mild DCM / Borderline ARVC	Beta-blocker
R	M	DCM	Dyspnea (52)	6	LBBB AV-block (1st)	No	.	35%	56 mm RWMA LGE+	.	.	-	-	-	-	+	27,8	DCM Death due to heart failure	*LMNA* c.1879C>T

Supplementary Table 2. Individual *LMNA* p.(Arg331Gln) carrier characteristics ordered by pedigree. (*Continued*)

S	M	DCM	Syncope (42)	0	No	No	VT	37%	62 mm	Normal		-	-	-	-	+	-	DCM	LDB3 c.1885G>A
T	M	DCM	DCM (50)	4	LBBB	pAF	VF	30%	71 mm	Impaired	.	-	-	-	-	-	19,4	DCM ICD HTx	
U	F	DCM	DCM (48)	7	AV-block (2nd)	pAF	No	20%	52 mm	Impaired	.	-	-	-	-	-	26,3	DCM ICD LVAD, HTx	
V	F	VT and sudden cardiac death in family	Syncope (31)	5	Low voltage	pAF	VT	48%	56 mm	64%	.	-	-	-	-	+	33,5	VA, ICD	DSC c.942+3A>G Calcium antagonist ACE inhibitor Diuretic
W	M	VT and possible DCM	AV-bock (2nd) (48)	13	AV-block (2nd)	AF	VT	35%	57 mm	Normal	-	-	-	-	-	-	24,9	DCM PM, ICD ATP therapy	Beta-blocker ACE inhibitor

Probands displayed in bold; *Measurements are based on lowest measured ejection fraction and largest end-diastolic diameter; †Left ventricular end-diastolic volume > 95th percentile indexed for body surface area, age and sex measured with MRI. ACE = Angiotensin-converting enzyme; AF = Atrial fibrilliation; ARVC = Arrhythmogenic right ventricular cardiomyopathy; Appr. = appropriate; ATrR = Angiotensin receptor ATP = Antitachycardia pacing; AV = Atrioventricular BMI = Body mass index; CAD = Coronary artery disease; CCD = Cardiac conduction delay; CRT-D / P = Cardiac resynchronization therapy defibrillator / pacemaker; CVA = Cerebrovascular accident; DCM = Dilated cardiomyopathy; DM = Diabetes Mellitus; kg/m2 = kilograms/meter2; ECG = Electrocardiogram; EF = Ejection fraction; F = Female; HTx = Heart transplant; ICD = Implantable cardioverter-defibrillator; CM = Cardiomyopathy; Inhom. = Inhomogeous; IV cond. = Intraventricular conduction; LAHB = Left anterior hemiblock; LBBB = Left bundle branch block; LVEDD= Left ventricular end diastolic dimension; LVAD = Left ventricular assist device; LVF = Left ventricular function; LGE = Late gadolinium enhancement; M = Male; MI = Myocardial infarction; mm = millimeter; NCCM = Non-compaction cardiomyopathy; PA = Pathology; pAF = Paroxysmal atrial fibrillation; PAC = Premature atrial contractions; PLD = Partial lipodystrophy; PM = Pacemaker; Pron. trab. Pronounced trabecularisation; PVCs = Premature ventricular complexes; NSVT = Non sustained ventricular arrhythmia; Presympt. = Presymptomatic; RBBB = Right ventricular bundle branch block RVEDD = Right ventricular end diastolic dimension; RWMA = Regional wall motion abnormalities; SND = Sinus node dysfunction; SVA = Supraventricular arrhythmia; Sympt. = Symptomatic; TFC2010+ = Fulfilling major criterion structural alteration right ventricle according Task Force Criteria 2010; VA = Ventricular arrhythmia; VF = Ventricular fibrillation; Yrs. = Years; 24H = 24 Hours.

Supplementary Table 3. Overview of *LMNA* mutations carriers (30 probands and 26 family members) included in survival analysis.

Mutation	Genomic position (GRCh37)	Number of probands	Number of Family members	Indication genetic screening probands
c.73C>T, p.(Arg25Cys)	156084782C>T	1	1	DCM
c.250G>A, p.(Glu84Lys)	156084959G>A	1	5	DCM
c.466C>A, p.(Arg156Ser)	156100517C>A	1		DCM
c.481G>A, p.(Glu161Lys)	156100532G>A	2		DCM
c.514-1G>A	156104193G>A	1	3	DCM
c.547C>T, p.(Leu183Pro)	156104227C>T	1		DCM
c.568C>T, p.(Arg190Trp)	156104248C>T	1		DCM
c.569G>A, p.(Arg190Gln) and c.746G>A, p.(Arg249Gln)	156104249G>A	1		DCM
c.624-626delGAA, p.(Lys208del)	156104304delGAA	1	3	DCM + LGMD
c.746G>A, p.(Arg249Gln)	156104702G>A	2		DCM
c.777T>A, p.(Tyr259*)	156104733T>A	1	2	DCM
c.855delG, p.(Ala287Leufs*193)	156105026delG	1		DCM
c.936+1delG	156105104delG	1		DCM
c.936+2T>G	156105105T>G	1		DCM
c.1004G>A, p.(Arg335Gln)	156105759G>A	1		DCM
c.1045C>T, p.(Arg349Trp)	156105800C>T	1	1	DCM
c.1130G>A, p.(Arg377His)	156105885G>A	1	1	DCM + EDMD
c.1130G>T, p.(Arg377Leu)	156105885G>T	2	3	DCM
c.1157+1G>A	156105913G>A	1		DCM
c.1157+46delC	156105958delC	1		DCM
c.1370delA, p.(Lys457Serfs*23)	156106217delA	1		DCM

Supplementary Table 3. Overview of *LMNA* mutations carriers (30 probands and 26 family members) included in survival analysis. *(continued)*

Mutation	Genomic position (GRCh37)	Number of probands	Number of Family members	Indication genetic screening probands
c.1380+1G>A	156106228G>A	2	1	DCM
c.1513_1514insGA, p.(Thr505Argfs*44)	156106228G>A	1	1	DCM
c.1517A>C (p.His506Pro)	156106928_156106929insGA	1		DCM
c.1608+4A>G	156106932A>C	1	4	DCM
c.1-318-?_c.356+?del (del exon 1)	156107027A>G	1	1	DCM

DCM = Dilated cardiomyopathy; EDMD = Emery-Dreifuss Muscular Dystrophy; LGMD = Limb-girdle muscular dystrophy.

Supplementary table 4. Overview characteristics of *LMNA* p.(Arg331Gln) carriers and pathogenic *LMNA* mutations carriers other than the *LMNA* p.(Arg331Gln) included in survival analysis**.**

	***LMNA* p.(Arg331Gln)**	***LMNA* group**	**P-value**
Male	61% (35/57)	50% (28/56)	0.258
Proband status	40% (23/57)	55% (31/56)	0.133
Hypertension	23% (12/52)	20% (10/50)	0.811
Dyslipidemia	8% (4/50)	16% (8/50)	0.234
Diabetes Mellitus type 2	6% (3/50)	8% (3/52)	1
Coronary artery disease	8% (4/51)	0% (0/35)	0.142
Anti-arrhythmics	67% (33/49)	57% (30/53)	0.419
ACE inhibitor or ARB	57% (28/49)	59% (31/53)	0.844
Diuretics	43% (21/49)	51% (27/53)	0.431

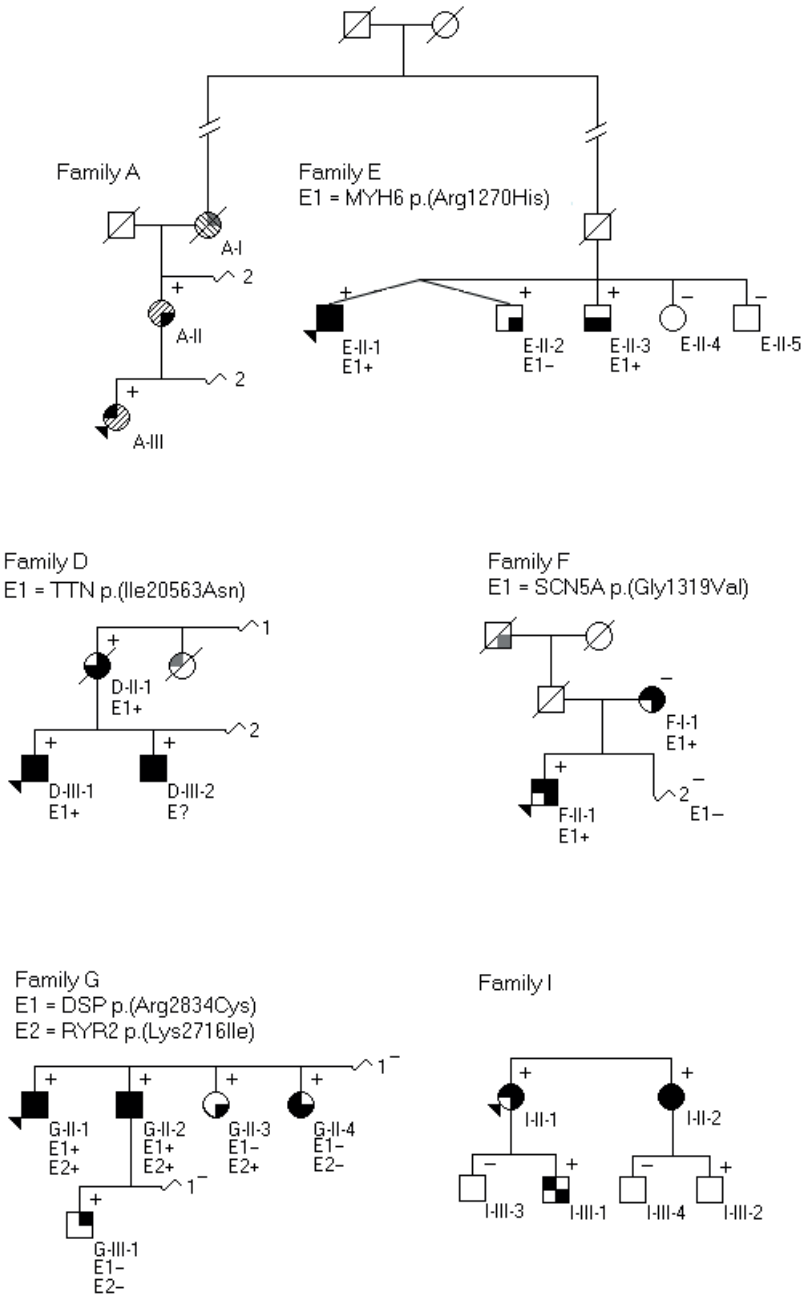
Supplementary Table 5. Shared haplotype surrounding the *LMNA* gene in p.(Arg331Gn) probands

		Probands															
Marker	Position	A*		E*		B		D		F		I		L		P	
D1S305	154,3	167	159	167	167	167	165	167	169	167	161	167	167	167	157	163	163
D1S2714	155,1	238	234	238	238	238	238	238	234	238	234	238	238	238	238	238	242
D1S1153	155,3	306	306	306	278	306	306	306	306	306	298	306	302	306	274	306	278
D1S303	155,6	183	189	183	179	183	183	183	187	183	187	183	183	183	179	183	187
D1S1595	155,7	287	275	287	291	287	279	287	275	287	275	287	287	287	279	287	287
LMNA p.(Arg331Gln)	156,1	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
D1S2624	156,6	207	203	207	203	207	203	207	203	207	201	207	203	207	207	207	201
D1S2625	157,5	422	422	422	422	412	420	412	414	422	422	422	424	422	422	426	420
D1S2626	158,0	212	210	212	218	212	212	212	214	212	214	212	214	212	212	212	212
D1S2627	158,5	310	304	302	312	302	300	302	302	302	300	302	300	306	298	314	308
D1S2635	159,2	146	138	142	142	150	144	142	142	150	146	152	146	152	146	146	154

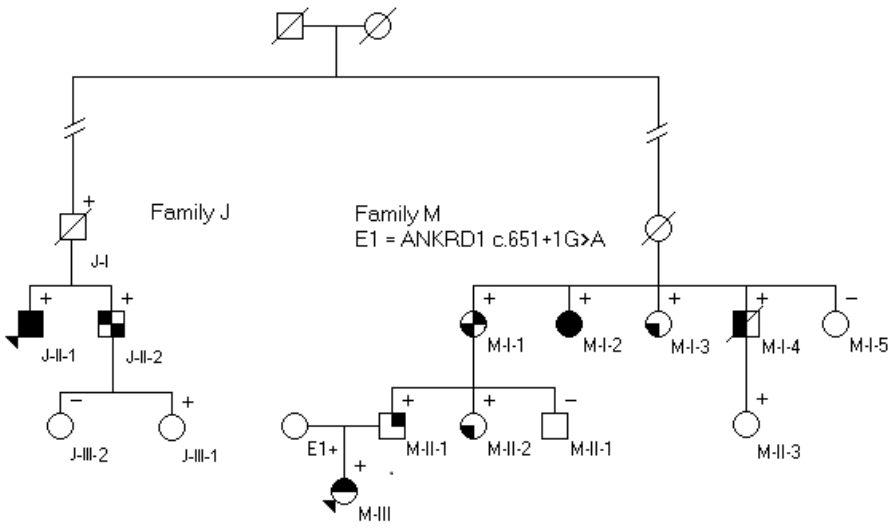
		Probands													
Markers	Position	C		G		J†		M†		K		N		O	
D1S305	154,3	169	159	167	167	167	157	167	165	167	161	167	159	167	153
D1S2714	155,1	238	234	238	240	238	238	238	234	238	242	238	238	238	240
D1S1153	155,3	310	310	310	310	310	302	310	306	310	286	310	298	310	286
D1S303	155,6	183	183	183	183	183	181	183	179	183	179	183	181	183	183
D1S1595	155,7	287	283	287	283	287	283	287	291	287	283	287	283	287	283
LMNA p.(Arg331Gln)	156,1	+	-	+	-	+	-	+	-	+	-	+	-	+	-
D1S2624	156,63	207	203	207	201	207	207	207	203	207	203	207	205	207	201
D1S2625	157,54	422	422	422	422	422	414	422	418	422	424	422	422	422	414
D1S2626	158,0	212	214	212	210	212	214	212	220	218	218	212	214	212	212
D1S2627	158,57	310	298	302	306	300	300	300	298	302	300	302	296	302	300
D1S2635	159,2	158	142	150	152	152	150	146	140	150	144	150	154	152	138

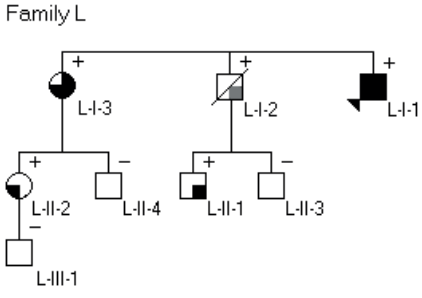
Marker D1S1153 (light grey) differs in approximately one half of the probands. It is likely that this has occurred due to slippage during replication some time ago. All probands share a common haplotype of approximately 1 Mb around the *LMNA* gene. *Families A & E have been genealogical linked and share common ancestors 6 generations back. †Families J & M have been genealogical linked and share common ancestors 6 generations back. Dark grey = phase of microsatellite markers.

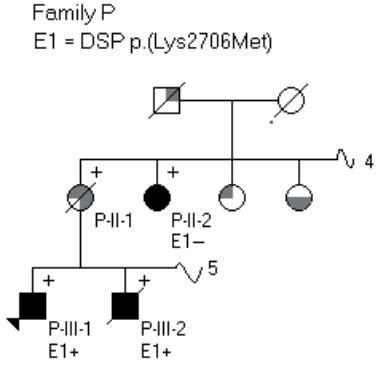
Supplemental Figure 1. Overview Pedigrees

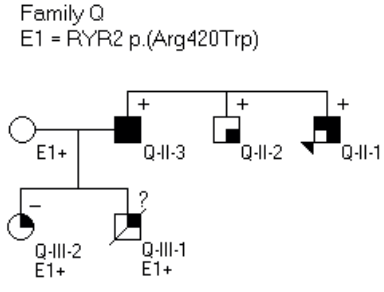


Supplemental Figure 1. Overview Pedigrees *(continued)*

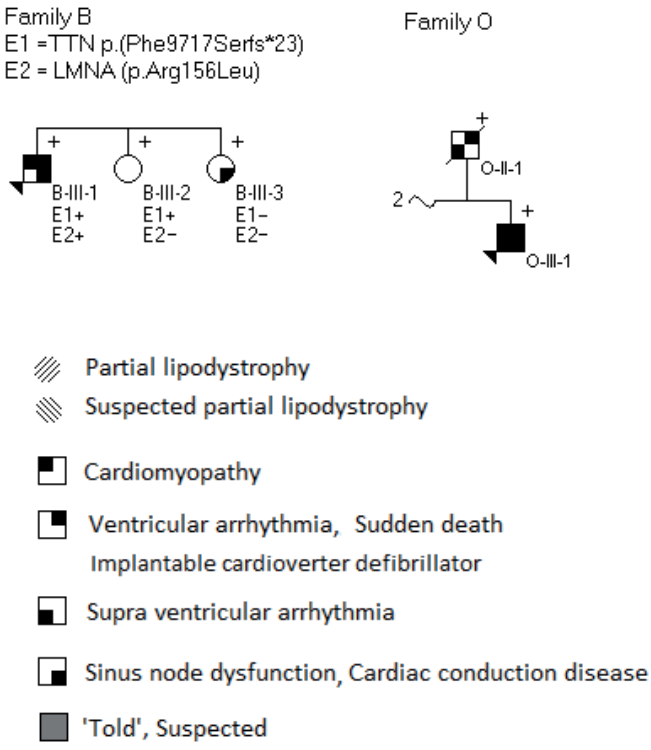








Supplemental Figure 1. Overview Pedigrees *(continued)*



The mutation segregates with the phenotypes typical for *LMNA* mutations. Only in family F is the segregation not clear. In this family, the phenotype of the mother of the index is probably caused by the pathogenic SCN5A p.(Gly1319Val) mutation.

Supplemental References:

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